

EVALUATION ON THE EFFECTS OF ACALYPHA WILKESIANA IN DRUG RESISTANT PATHOGENS

Submitted in partial fulfilment of the

Requirements for the award of

Master of Science

In

Medical Biotechnology and Clinical Research

by

Ms. KAVYASHREE V (Reg No: 41101005)



**CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES INTERNATIONAL
RESEARCH CENTRE**

SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600119

MAY- 2023



SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited "A" Grade by NAAC | 12B Status by UGC | Approved by AICTE

www.sathyabama.ac.in

CENTRE FOR MOLECULAR AND NANOMEDICAL SCIENCES

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **Ms. KAVYASHREE V** (41101005) who carried out the project entitled "**EVALUATION ON THE EFFECTS OF ACALYPHA WILKESIANA IN DRUG RESISTANT PATHOGENS**" under my supervision from October 2022 to April 2023.

for Dr. R. Rajesh Kannan

GUIDE

HEAD OF THE DEPARTMENT

Submitted for Vivo voce Examination held on 05.05.2023

S. John Penny Samuel
Internal Examiner 05/05/23

K. C. S. J.
External Examiner 5/5/23

DECLARATION

I, **Ms. KAVYASHREE V** (Reg No: 41101005) hereby declare that the Project Report entitled “**EVALUATION ON THE EFFECTS OF ACALYPHA WILKESIANA IN DRUG RESISTANT PATHOGENS**” was successfully done by me under the guidance of **Dr. R. Rajesh Kannan** submitted in partial fulfilment of the requirements for the award of Master of Science Degree in Medical Biotechnology and Clinical Research.

Date: 05.05.2022

Place: CHENNAI



Signature of the candidate

ACKNOWLEDGEMENT

First and foremost, I would express my sincere gratitude to our Honorable Founder Chancellor **Col. Dr. JEPPIAAR, M.A., B.L., Ph.D.**, For providing me the necessary facilities for the completion of my Project.

I feel greatly indebted and glad to express my sincere thanks to Chancellor **Dr. Maria Zeena Johnson, B.E., M.B.A., M.Phil., Ph.D.**, for providing me the necessary facilities for the completion of my Project I also thank my President **Dr. Marie Johnson, B.E., M.B.A., M.Phil., Ph.D.**, and Vice presidents **Mr. J. Arul Selvan and Ms. Maria Bernadette Tamilarasi Johnson** for their constant support and endorsement.

I would like to express my gratitude to Vice Chancellor **Dr. T. Sasi Praba, Ph.D.**, Sathyabama Institute of Science and Technology for having been a constant source of support and encouragement for the completion of the Project.

I also thank Registrar **Dr. S. S. Rau, M.B.A., Ph.D.**, and Controller of Examinations **Dr. S. Igni sabasti prabu M.E., Ph.D.**, for providing me with the necessary facilities for the completion of the Project.

I would like to offer my special thanks to my Course Coordinator **Dr. A. Dayanandan**, Scientist – E, Centre for Molecular and Nanomedical sciences, for pursuing me to do Project and allocating mentor to carry out my project.

I wish to extend my special thanks to my Guide of the Project **Dr. R. Rajesh kannan**, M.Sc., Ph. D., Scientist – D, Centre for Molecular and Nanomedical sciences and all scientists and scholars in the department for the constant encouragement and guidance throughout this Project.

I would also like to express my sincere thanks to the research scholars **Mr. W.A Carlton Ranjith, Mr. S. Nandhagopal and Ms. A. Dhanalakshmi**, Centre for Molecular and Nanomedical sciences for helping me to carry out the project work.

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are one of the most significant Multidrug resistant infections, which has become a severe danger to public health on a global scale. Also, the healthcare industry is greatly impacted by biofilms. Bacterial biofilms naturally exhibit antimicrobial resistance, and MRSA is more likely than other strains to adhere to biotic and abiotic surfaces and form biofilms, which develops a chronic infection. As a result of resistance to several prevailing antibiotics, a new kind of antibacterial agents with novel mechanisms of action are still highly sought-after on a worldwide scale. Numerous antibacterial substances, including lactams, polyketides, phenylpropanoids, aminoglycosides, macrolides, glycopeptides, streptogramins, and lipopeptides, are either natural products or semisynthetic variations of those substances. Many medicinal chemists have been intrigued by the prospect of revisiting natural products with contemporary chemistry and biology tools in order to discover and develop natural products or derived semisynthetic derivatives as efficient antibacterial agents in the context of the alarming rise in antibacterial resistance. Studies suggested that using *Acalypha wilkesiana* leaves in ethnomedicine for the possible management of skin conditions, gastrointestinal problems as well as other related microbial infections. Therefore, the purpose of the current investigation was undertaken to elucidate the antibacterial activity of the leaf extract of *A. Wilkesiana* and to determine its anti-biofilm activity, which could be used as a potential drug candidate against MRSA infections.

Keywords: Methicillin resistant staphylococcus aureus (MRSA), *Acalypha wilkesiana*, antibacterial, anti-biofilm.

TABLE OF CONTENTS

S.NO	TITLE	PAGE NUMBER
1	List of abbreviation	vii
2	List of Tables	vii
3	List of figures	viii
4	Introduction	1
5	Review of literature	7
6	Aim and objective	10
7	Materials and methods	11
8	Results	17
9	Summary and conclusion	28
10	References	29

LIST OF ABBREVIATION

AMR - Antimicrobial resistance

MRSA - Methicillin resistant *Staphylococcus aureus*

CDC - Center for Disease Control and Prevention

RSS - Risk Stratification-based Surveillance

A.W - *Acalypha wilkesiana*

PBS - Phosphate Buffer Saline

TMTC - Too Many To Count

TFTC - To Few To Count

NOAEL - No Observed Adverse Effect Level

NOEL - No observed effect concentrations

LOAL - Lowest Observed Adverse Level

LIST OF TABLES

TABLE NUMBER	TITLE OF THE TABLE
4.1	Culturing Media and it's volume per 100ml of distilled water
5.1	Manual counting of colony forming units on different concentration of extract in different time intervals
5.2	ImageJ quantification of area on different concentration of extract in different time intervals
5.3	ImageJ quantification on area of biofilm

LIST OF FIGURES

FIGURE NUMBER	TITLE OF THE FIGURE
1.1	<i>Acalypha wilkesiana</i> plant
5.1	Process of leaf extraction by decoction method
5.2	Streaking of bacterial (MRSA) inoculum
5.3	Image of MRSA crystal violet staining by stereo microscope
5.4	Agar well diffusion method with serially diluted plant extract on MRSA
5.5	The minimum inhibitory concentration (MIC) graph
5.6	Time-kill of assay of <i>A. Wilkesiana</i> extract on different time intervals
5.7	Graph on the time kill kinetics of the extract in different time intervals
5.8	Area of MRSA colony on different concentrations of extract on different time intervals
5.9	3D Bar graph showing areas of MRSA Biofilm formation
5.10	Anti-biofilm forming capacity of the <i>Acalypha wilkesiana</i>
5.11	Development of zebrafish embryo to larvae, treated with highest concentrations of A.W extract
5.12	No observed effect concentrations (NOEC) in zebrafish larvae treated with serially diluted plant extract.

CHAPTER 1

INTRODUCTION

1.1. BACKGROUND

In most cases, nature permits coexistence between humans and microbes. Microorganisms have taken advantage from nature to express themselves in a variety of ways that are both helpful and harmful to mankind, acting as the primary cause of various infectious diseases that have afflicted people since the dawn of time and are still plaguing people today, becoming a major global crisis. Any illness brought on by microbial pathogens, such as fungi, viruses, and bacteria, that may spread from one organism to another, either directly or indirectly, is often referred to as an infectious disease (G V Kumar et al, 2016). Bacterial infections have been noted over the years as a substantial course of morbidities and are linked to high death rates. Alarming mortality rates have been reported throughout the world in several different geographic areas. According to recent reports, the Asian region has the highest rate of deaths worldwide (4,730,000 per year), followed by Africa (4,150), Latin America (392,000), Europe (390,000), North America (317,000), and Oceania (22,000). The World Health Organization (WHO) has identified antimicrobial resistance (AMR) as one of the top ten worldwide dangers to public health because of its recent upward trend (N Thomas et al, 2016).

Antibiotics were seen to be a medical marvel that had saved countless lives across the world. AMR has, however, increased internationally due to the overuse and misuse of these antibiotics in both human and animal medicine. AMR is a global public health problem because of the ongoing rise in drug-resistant diseases brought on by germs that have been found, treated, and identified (Basri, 2021).

There are two current main causes of mortality that are linked to bacterial infections. Sepsis has emerged as a serious threat to the general health of the populace around the world and is life-threatening due to organ dysfunction resulting from dysregulation in the host's immune reaction to infections. If unmasked and properly managed in the

early stages, sepsis could result in death. According to a recent analysis by Pant et al, bacterial infections are a leading cause of sepsis, which has been estimated to have 48 million recognized cases and an 11-million-person fatality rate globally. Broad-spectrum antibiotics that have been shown to be efficient in eradicating the causing microorganisms are used as part of the therapeutic mitigation of this illness condition, with emphasis placed on source. (J S Kim et al, 2011)

Additionally, there was a long-term and widespread steroid use during the most recent global pandemic, which decreased COVID-19 patients' immunity. Pan resistant infections developed as a result, necessitating the use of numerous or higher generation antibiotics like CIP. Additionally, the global spread of COVID-19 led to a rise in the misuse and consumption of antibiotics. (Akinyemi, 2005)

1.2 METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS

Gram-positive, clustered, spherical-shaped *Staphylococcus aureus* is predominantly a human and animal pathogen. *S. Aureus* is often prevalent in healthy people and doesn't infect healthy skin, but it can cause significant illnesses when it enters internal tissues or the circulation. Small skin infections brought on by *S. Aureus* include impetigo, scalded skin, pimples, boils, abscesses, etc. Additionally, it can lead to fatal conditions like sepsis, bacteremia, meningitis, pneumonia, and endocarditis. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious threat to people's overall health, whether they are in a hospital or a neighborhood setting. They have the ability, like many other bacteria, to protect themselves from the effects of medicinal medicines by creating a biofilm around themselves, which also protects them from the host's immune system's reaction.(Kranjec, 2020)

In 1961, MRSA was first identified. Numerous antibiotics, including methicillin, penicillin, oxacillin, cloxacillin, cefazolin, ceftiofur, and other widely used antibiotics, are ineffective against it. When in close proximity to an infected person, MRSA can spread. MRSA may spread from an infected object to a human or from one human carrier to another. Nosocomial infections, in general, refer to any infections acquired in hospitals or other healthcare facilities. HA- MRSA refers to MRSA infections that take place in hospital- or healthcare-related environments. CA-MRSA refers to MRSA infections that take place in

settings connected with the community. Skin-to-skin contact between healthy individuals can quickly spread it. Elderly or disabled persons living in cramped settings are frequently affected by CA-MRSA.(Mastoor,2022)

The MRSA epidemic extended throughout industrialized nations in the 1980s and 1990s as a result of the emergence of new clones. According to a study conducted by Hetem and colleagues, HA-MRSA clones seem to spread more often among hospitalized patients. In 2008, Iceland, Norway, and member states of the European Union (EU) reported approximately 380,000 HA-MRSA infections. The main clone associated with HA-MRSA infections is the Brazilian clone (ST239-III), and cases of it have been documented throughout Africa.(Basri, 2019)

Every country on earth has reported finding MRSA spreading. Currently, MRSA is pervasive in the majority of hospitals around the globe. According to CDC evaluations, MRSA has been labelled as a significant danger after considering a variety of characteristics, including the strain's prevalence, growing trends in resistance, treatability, mortality, preventability, and transmissibility, as well as the strain's effects on the community and healthcare infrastructure (Silva,2019). On the financial front, it is anticipated that treating MRSA infections will cost \$3 billion annually. Due to its successful clones, CA-MRSA has been the main MRSA infection for the past 10 to 20 years.

According to the Asian Network for Surveillance of Resistant Pathogens (ANSORP) research, MRSA infection rates in the Philippines, Thailand, and Vietnam between 2004 and 2006 were 38.1%, 57%, and 74.1%, respectively. According to another study, 18,000 patients died in the US in 2005. This number is greater than the proportion of HIV/AIDS-related deaths. (Iyekowa, O.,2016)

The Risk Stratification-based Surveillance (RSS) program's data collection in 2011 revealed the fact that the MRSA ratio among clinical *S. Aureus* isolates varied from 28% in Indonesia to 59% in the Philippines. MRSA infection rates in 2008–2009 were reported by two multicenter studies in India to be 41% and 45%, respectively. According to a 2020 article, MRSA frequently infects the skin and sensitive tissues. *S. Aureus*-related bacteremia has been linked to death rates of 15% to 60%. MRSA has no age

restrictions and may harm anybody, from infants to adults. If an MRSA bacteremia is not promptly diagnosed and treated, it may result in abrupt death. Up to 30% of youngsters have MRSA on their skin or noses, according to some US polls.

The Centers for Disease Control and Prevention (CDC) has, in a recent report, ruled MRSA-related infections and death (estimated to be 80,461 and 11,285, annually, respectively) as the arrowhead amongst other bacterial infections in the United States of America (USA) (Umamaheswari, A.,2020) . The high occurrence of MRSA has also been recorded across other continents, with Asia, South and North America recording over 50%; Europe 18 to 49%; and Africa and Australia 20 to 50% of recorded infections and bacteria-related diseases attributed to MRSA. Therefore, strategies to improve the treatment of the diseases caused by these bacteria are urgently needed.

Since all antibiotics share an inherent weakness as the bacteria, they target almost continuously developing new mechanisms of resistance on account of inadequate posology in hospital settings and communities. Therefore, there is an obvious need to counteract bacterial resistance. Research on chemical and biological properties of natural products over the past two centuries has resulted in the discovery of many drugs for the treatment of human diseases. More than 50% of all the drugs currently in use are of natural product origin. So, one way to fight resistance is the use of multi component therapy. Indeed, combination between two or more antibiotics or natural products with antibiotics broadens bacterial targets or increases the action on the same target. Berdy et al. Reported the presence of 200,000 bioactive natural products to maintain or boost the clinical effects of antibiotics via synergistic studies (Haruna,2013).

1.3 ACALYPHA WILKESIANA



Fig 1.1: *Acalypha wilkesiana* plant

Taxonomy

Kingdom – Plantae

Division – Spermatophyta

Class – Monochlamydae

Series- unisexuales

Family – euphorbiaceae

Genus – acalypha

Species – wilkesiana

Botanical name – *acalypha wilkesiana* Muell. Arg.

Common names

Acalypha wilkesiana is commonly called Irish petticoat. Other common names include copper leaf, Jacob's coat, Fijian fire plant, fire dragon plant, beefsteak plant, huddling, red leaf, and Joseph's coat.

Description

The Greek words for “without” and “cover ” in the name *Acalypha wilkesiana* are a and kaypohs, respectively. An evergreen shrub that is a member of the Spurge family and is thought to have originated in Fiji and the South Pacific Islands. Throughout tropical Africa, South Africa, India, Sri Lanka, America, Yemen, and Pakistan, as well as the

southern and central regions of Florida, the plant is widely distributed. It expands to a height of 3 meters (9.8 feet) and a width of 2 meters (6 feet 7 inches). The stem is upright and has several little, hairy branches. Its crown is compactly arranged. The leaves have a mottled look and are a coppery green tint with crimson accents. The edges of the broad, big leaves have teeth. Depending on the culture, they can range in size from 10 to 20 centimeters (3.9 to 7.9 in) long and 15 centimeters (5 to 9 in) broad, with alternating heart-shaped, elliptic to oval, serrate, hairy, and combinations of colors like green, purple, yellow, orange, pink, or white. They might be wrinkled or flat. At the ends of branches, there are spikes with crimson blooms. On the catkin-like racemes that grow beneath the foliage of the same plant, they have separate male and female flowers (Haruna, 2013). The female flowers are in small spikes, whilst the male blooms are in long spikes that hang downward. The flowers lack a nectar disc and have apetalous petals. Between February and December is when it blooms. It is planted as horticultural landscaping around houses. When regularly watered during a drought, it displays its greatest appearance. It can thrive in a wide range of garden soils with a minimum temperature of 10 °C. Any time of the year, it is easily propagated by stem cutting or air layering. Numerous cultivars with various leaf sizes, colors, and shapes have been created, and they thrive in rich, well-drained soil. *A. Wilkesiana* 'Godseffiana' has narrow, drooping, green leaves with creamy-white margins, 'Marginata' has coppery-green leaves with pink or crimson margins, 'Macrophylla' has larger leaves, variegated with bronze, cream, yellow and red, while 'Musaica' has green leaves that are mottled with orange and red (Lee, et al, 2018).

Phytochemistry

The phytochemical screening of the leaf extract identified the presence of saponins, tannins, alkaloids, phlobatanins, phenolics, and cardiac glycosides. Sesquiterpenes, monoterpenes, triterpenoids, polyphenols, gallic acid, corilagin, geraniin, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, saponins, tannins, anthraquinone, and glycosides were discovered in *Acalypha wilkesiana* leaves in earlier analyses (Akinde, 1986; Adesina et al. They were also shown to be abundant in several powerful

hepatoprotective substances, such as tannic acid, flavonoids, and vitamin C (Shrira, et al, 2013).

Antimicrobial activities

Acalypha wilkesiana has been said to possess antibacterial and antifungal qualities in addition to being used as a vegetable. According to Awe and Eme et al (2014), ethanolic extracts of *A. Wilkesiana* have antibacterial properties and prevented the development of both gram positive (*S. Aureus*) and gram negative (*E. Coli*, *K. Pneumonia*, and *Shigellasp.*) bacteria.

Additional research on plant extract fractions has revealed bactericidal activities as well as inhibitory effects on the development of methicillin-resistant *Staphylococcus aureus*. The emulsifying ointment base-containing formulation of *Acalypha wilkesiana*'s ethanol extract showed superior antibacterial and anti-candidal activities. Olaribigbe and Sorbari (2014) claim that *Acalypha wilkesiana* creams made with cetomacrogol have the most antibacterial effects, whilst emulsifying ointments have the weakest effects. This is because the formulation's inclusion of paraffin has an inhibitory impact. As a result, the traditionalists' assertion that the *A. Wilkesiana* leaf has antibacterial properties to treat skin infections is true, and it may be utilized to make herbal skin lotions to treat various skin illnesses. The antibacterial activity of *Acalypha wilkesiana* extracts on the gastrointestinal tract is attributed to triterpenoids of the cyclooctane-type, flavonoids like quercetin- and kaempferol-derivatives.(Nwabuife, et al , 2022)

Thus, in this study we aimed to evaluate the antibacterial and anti-biofilm activity of the plant extract of *Acalypha wilkesiana* using water as solvent. Thereby providing positive observation that could promote an alternative promising treatment for the human selective pathogenic organism MRSA.

CHAPTER 2

REVIEW OF LITERATURE

Algammal, 2020 said that one of the biggest concerns to public health in the twenty-first century is the development of bacterial antibiotic resistance, which happens when changes in bacteria make the medications used to treat diseases less effective. According to the UK Government-commissioned Review on Antimicrobial Resistance, by 2050 AMR might kill 10 million people annually. Although some have criticized these projections, the WHO and numerous other organizations and researchers concur that the spread of AMR is an urgent problem that needs to be addressed through a global, coordinated action plan. It is essential to know the top pathogen-drug combinations contributing to the burden of bacterial AMR, trends throughout the world, and the present size of the problem. If AMR continues to develop unchecked, many bacterial infections might become considerably more deadly than they are now in the future.

Mabrouk,et al 2020 Understanding the full cost of resistance is a difficult task when trying to combat AMR, especially in areas with poor monitoring and data. There is abundant literature that estimates the effects of AMR for pathogen-drug combinations in particular locations on incidence, deaths, hospital length of stay, and health-care costs. However, to our knowledge, no comprehensive estimates covering all locations and a wide range of pathogens and pathogen-drug combinations have ever been published. For instance, Cassini and colleagues estimated the burden of eight bacterial pathogens and sixteen pathogen-drug combinations in the EU and European Economic Area for 2007–15, while the US Centers for Disease Control and Prevention published a 2019 report on AMR infections and deaths in the USA for 18 AMR threats using surveillance data. Similarly, Temkin and colleagues estimated the prevalence of *Escherichia coli* and *Klebsiella pneumoniae* resistant to third generation cephalosporins and carbapenems in 193 countries in 2014 and Lim and colleagues estimated the burden of multidrug resistance in six bacterial pathogens in Thailand in 2010,11.

Atoum, et al, 2003 stated that although these publications make significant contributions to the body of research on AMR, they are not enough to comprehend the global burden of AMR or to recognize and target the pathogens that pose the greatest threats in various regions. Additionally, most previous studies have only taken into account one type of AMR burden. It is crucial to calculate the burden based on both of these counterfactual scenarios because we are unsure of the extent to which drug-resistant infections would be replaced by susceptible infections or by no infections in a situation in which all drug resistance was removed.

Pantosti, A., et al 2009, presented a work on Methicillin-resistant staphylococcus aureus (MRSA), responsible for the most frequent cause of nosocomial infections, which is regarded as a major human pathogen. Infections of the skin and soft tissues as well as more dangerous invasive infections such pneumonia, bacteraemia, endocarditis, and osteomyelitis are all brought on by MRSA. Over the past few years, there has been a significant rise in MRSA infections, which are more frequently linked to mortality than other bacterial infections. Despite receiving the right care, *S. Aureus* has a 20–40% death rate after 30 days. Patients are at significant risk from MRSA because it can form biofilm. The organized structure created by practically all bacteria, biofilm, which is made up of nucleic acids, lipids, proteins, and polysaccharides, is regarded as a primary virulence factor. *S. Aureus* is regarded as the main species in biofilm-associated illnesses, and biofilms are involved in more than 80% of human infections. MRSA, like other bacteria, exhibits enhanced persistence in the body, environment, and on medical surfaces in Biofilm, as well as increased resistance to medicines and human immunological responses, which is a significant medical issue. As a result, the discovery of new chemical compounds to cure MRSA infections and their biofilm is urgently needed.

Gajdács, Márió, 2019 stated that Plant extracts and their essential oils (EOS) that act as antibacterial and also against bacterial biofilm are of tremendous interest. Natural products were the first ever cure available to mankind in history as no synthetic medicines were yet discovered then. As a result, the use of plant natural products in drug discovery is highly significant due to two factors: first, natural products explore a

different region of the chemical space than synthetic compounds, and second, natural products are receptive to further development. The limitations of natural product research, such as the lengthy screening, isolation, and structure elucidation processes as well as the difficult compound development, have caused pharmaceutical companies to reduce their funding and interest over the past few years. However, as technology advances and the challenges of the natural product approach to drug discovery are overcome, there has recently been a resurgence of interest.

The name *Acalypha wilkesiana* originated from Greek, *a*, which means “without” and *kaypohs*, which means “cover”. The plant was named after Admiral Charles Wilkes, a 19th century American naval officer and explorer in The South Pacific.

A Wilkesiana is a tropical bush with ovate to elliptic-shaped, variegated copper-red leaves. Its 10 to 20 centimetre long leaves are arranged in a straightforward spiral pattern. This plant, which may reach a height of 4 meters, is grown as an ornamental plant in the Asia-Pacific area. The spike-like inflorescences have male and female flowers at their bases. The flowers lack a nectar disc and have patulous petals. The plant flowering season is from February to December.

Lakhundi, et al 2019 stated that the leaves are traditionally used in treatments for inflammation, headache, fever and flu, whereas their roots are boiled to wash haemorrhoids. A beverage consisting of about 60g of leaves boiled in goats’ milk is drunk to lower blood temperature, treat fever, relieve cough and heal pimples. Although the complete pharmacological properties of *A. Wilkesiana* are unknown, some evidence have been produced on *A. Wilkesiana* showed that the phytochemical and biological examination of the leaves, which observed a broad-spectrum inhibition of Gram-positive and Gram-negative organisms. Indeed, water and ethanol extracts of this plant showed antimicrobial properties with standard and local strains of Gram-positive bacteria *Staphylococcus aureus* and A study in vitro on Methicillin Resistant *Staphylococcus aureus* (MRSA) reported good antibacterial activity against MRSA.

CHAPTER 3

AIM AND OBJECTIVE

3.1 AIM

To evaluate the anti-bacterial activity and anti-biofilm activity of the *Acalypha wilkesiana* plant extract on a drug resistant pathogen- MRSA.

3.2 OBJECTIVE

- To obtain the leaf extract of the plant *Acalypha wilkesiana* (red) using water as solvent by decoction method followed by lyophilisation
- To obtain a pure culture of well-isolated colonies of MRSA
- To evaluate the effects of *A.wilkesiana* on MRSA and study its anti-microbial and anti-biofilm activities.
- To study the toxicological effects of the extract on the zebrafish embryo and larvae
- To screen the compounds, present in plant extract and docking to find the best interacting compound

CHAPTER- 4

METHODOLOGY

4.1 PREPARATION OF PLANT EXTRACT

The test plant *Acalypha wilkesiana* (red) was purchased from a local garden. The extraction was done by decoction method where 30 grams of fresh leaves with 300 ml water (1:10 ratio) were boiled continuously for 30 minutes and filtered with Whatman no 1 filter paper. The filtrate was concentrated using rotary evaporator under reduced pressure at 40°C. The liquid samples were placed in petri dishes (7mm), then frozen at -20°C and finally lyophilized at -55°C under vacuum for 12 hours.

4.2 BACTERIAL CULTURE

Media preparation

Table 4.1: *Culturing Media and it's volume per 100ml of distilled water*

Media	Volume per 100 ml dH ₂ O
Nutrient agar	2.8g
Nutrient broth	1.3g
Mueller Hinton broth	2.1g
Tryptic Soy Broth	3.0g

PBS Preparation

NaCl (0.8g), KCl (0.02g), NaHPO₄ (0.144g), KH₂PO₄ (0.024g) was dissolved in 100 ml distilled water (pH-7.4)

Sterilization: Autoclaved at 121°C for 15 minutes

Materials: Media, sterilized petri plates, test tubes, Bio-safety cabinet, bacterial strain, inoculation loop, etc.,

4.2.1 Streak plate method

Principle

It is based on dilution during the process of mechanical spreading of inoculum over the surface of solidified culture media in order to obtain well-isolated colonies of the sample at the terminal streaks

Objective

- To obtain a pure culture of bacteria from a mixed culture
- To obtain well-isolated colonies
- To propagate bacteria

Quadrant streaking procedure

- i. The petri dish was held at a 60° angle
- ii. Using a swift, gentle, back and forth motion, the sample was distributed across approximately $\frac{1}{4}$ th of the media in the petri plate.
- iii. The loop was sterilized and allowed to cool. The loop was streaked from the last streaks of the first quadrant after rotating the petri plate 90° anticlockwise. Moved the loop back and forth to disperse the cells.
- iv. The inoculum across the remaining half of the first quadrant's streaks into the second quadrant's empty space. Steps were repeated to streak the third and fourth quadrants.
- v. In the fourth quadrant, the final streak is formed in a zigzag pattern to form a tail.

4.2.2 GRAM STAINING

It is the common, important differential staining techniques which helps in the classification of gram positive and negative bacteria

Principle

Gram positive bacteria have a strong wall made of protein-sugar mix and have less fat. When the color is removed, the wall dries up and becomes smaller, closing the holes in it. This prevents the stain from leaving the cell. Therefore, the ethanol cannot take away the stain bound to the peptidoglycan of gram-positive bacteria and appears blue or purple in color

Reagents

crystal violet, the primary stain

iodine, the mordant

a decolourizer made of acetone and alcohol (95%)

safranin, the counterstain

Procedure

- i. The bacterial suspension was smeared on a clean glass slide, air dried and heat fixed
- ii. Crystal violet was poured and kept for about 30 seconds to 1 minute and rinsed with water
- iii. The gram's iodine was flooded for 1 minute and washed with water
- iv. Then, the slide was washed with 95% alcohol 10-20 seconds and rinsed with water, air dried, blot dried and observed under stereomicroscope.

4.2.3 MRSA culturing procedure

The strain Methicillin Resistant Staphylococcus Aureus, pure isolates were from IBMS and was cultured on nutrient broth and nutrient agar plates by streak plate method and confirmation is done by the gram staining process as described in the Textbook of Diagnostic Microbiology. The bacterial isolates were maintained on the nutrient agar and broth at 4°C.

4.3 AGAR WELL DIFFUSION METHOD

The agar well diffusion method is used to study antibacterial activity of the plant extract and to determine its susceptibility or resistance of the bacterial strain. For this method, 6mm well was bored on the agar plate containing isolate that equals 1×10^6 CFU/ml. A stock solution of the extract was prepared by dissolving 0.5 grams of extract in 5ml of the solvent (water) to produce the final concentration of 100 mg/ml. The stock solution was then serially diluted to concentrations of 0.75, 1.5, 3.12, 6.25, 12.5, 25, 50 and 100 mg/ml. 20 μ l of each dilution was inundated in each wells along with amoxicillin and potassium clavulanate as positive and water is used as the negative control and incubated for 24 hours. Antibacterial activity was evaluated by measuring the diameter (mm) of the inhibition zone (ZOI) around the wells produced by the leaf extract.

4.4 BROTH MICRODILUTION METHOD

The growth inhibition assay is performed as per the CLSI guidelines, where each well of the 96-microtiter plate was filled with 100 μ l of MH broth. 100 μ l of the stock solution (100 mg/ml) of plant extract was added on each well and serially diluted within the range of 0.75 to 100 mg/ml. The bacterial suspension was adjusted to 0.5 McFarland standard (1×10^8 CFU/ml) and further diluted and adjusted to the appropriate inoculum size of 1.0×10^6 CFU/ml. A volume of 10 μ l of the MRSA suspension was dispensed into each well, labelled appropriately. For the growth control only the bacterial suspension with the growth media is added and sterility control (blank) only the MHB is added. After 24 hours of incubation at 37°C, aliquots (10 μ l) were taken from each well and inoculated into freshly prepared 200 μ l MH broth incubated at 37°C for 24h for confirmation. Also, the microtiter plate is read by the ELISA plate reader at 600nm. The procedure was carried out in independent triplicates to validate the results.

4.5 TIME KILL ASSAY

The time kill kinetics of the plant extract *A.wilkesiana* was carried out by following the procedures described by Tsuji et al. Concentrations equals to the half the MIC, MIC, twice the MIC and thrice the MIC of the plant extract was prepared. An inoculum size of 1.0×10^6 CFU/ml was added and incubated at 37°C. Aliquots of 20 µl of the medium were taken at the time intervals of 0,2,4,6 and 24 hours, incubated aseptically into 20 mL nutrient agar and incubated at 37°C for 24h. A control test was performed for the organism without the extract. The Colony forming units were determined manually and image processing was done by the software ImageJ. The procedure was performed in triplicate and a graph of the log CFU/mL was plotted against time.

4.6 ANTI-BIOFILM ACTIVITY STUDY

The MRSA was grown on Tryptic-Soy Broth (TSB) with 0.5% glucose for 24h in order to prepare cell suspension containing about 1×10^8 CFU/mL matching the 0.5% Mc Farland standard solution. Subsequently, about 50 µl of the MRSA cell suspension was inoculated in six sterile glass slides. Biofilms were developed for 48h at 37°C. After this time, the medium was removed, and non-adherent bacterial cells were discarded by washing twice with sterile Phosphate buffer saline (PBS, pH 7.4). The biofilms were fixed with 100 µl of methanol per slide for 15 minutes and stained for 5 mins with 100 µl of 1% crystal violet per slide. After rinsing with water the slides were air dried at room temperature. For control the slides were observed under light microscope and quantified the biomass of the biofilm with the software ImageJ. For anti-biofilm activity of the formed biofilms were treated with the extract of concentrations equal to MIC, 2X MIC, 3X MIC and each assay was performed three times and the results were averaged.

4.7 TOXICITY STUDIES

Plant extract stock preparation:

1 mg of the *A.wilkesiana* extract was dissolved in 10 ml of fresh water making a concentration of 100 µg/ml.

E3 media preparation:

- i. To prepare 60X stock E3 media, NaCl (1.74g), KCl (0.08g), CaCl₂ (0.29g) and MgCl₂ (0.489) were dissolved in 100 ml of distilled water (adjusted to 7.2 pH).
- ii. To prepare 1X E3 medium, 1ml of the stock solution was added to 59ml of fresh water and a pinch of 1% ethylene blue was added in it.

Toxicity test procedure:

Zebrafish Embryo Acute Toxicity Test Method was used to determine the toxicity of the plant extract following the OECD guidelines. ZFET was carried out with the newly fertilized eggs from zebrafish (*Danio rerio*). In the 24 well plates, 20 live embryos were added in each well containing 1ml E3 media. The plant extract was serially diluted from a range 0.75 µg/ml to 100 µg/ml and 1 ml of each concentration was dispensed in the wells accordingly. Also, a control was maintained containing only the embryos with E3 medium. After 24 hours, the plate was observed for the presence of deformities (removed if present) and the observations were noted. After hatching (48 hours) the larvae were observed under the stereomicroscope and analyzed for the effects of the plant extract.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 PLANT EXTRACTION

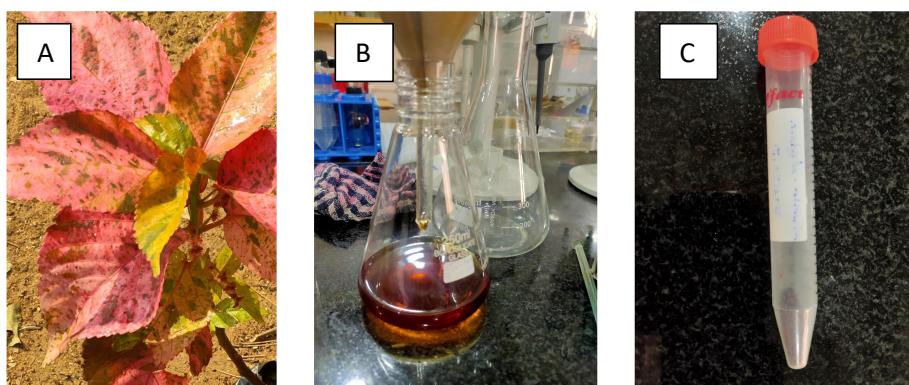


Figure 5.1: Process of leaf extraction by decoction method

(A) *Acalypha wilkesiana* plant

(B) Decoction of the *A. Wilkesiana* leaf

(C) Lyophilized powder extract

From the 30 grams of leaves of *A. Wilkesiana* and 300 ml of solvent water (1:10 ratio) 100ml decoction was obtained and concentrated so that the volume reduced to approx. 50ml. After extraction by lyophilization around 1.3 grams of *Acalypha wilkesiana* powderd extract was obtained.

5.2 MRSA CULTURE

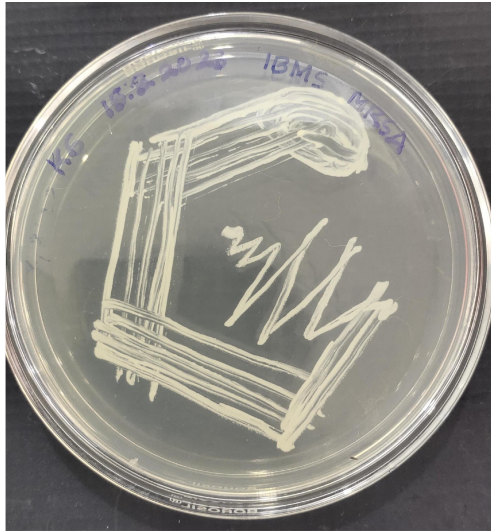


Figure 5.2: Streaking of bacterial (MRSA) inoculum

5.3 GRAM STAINING

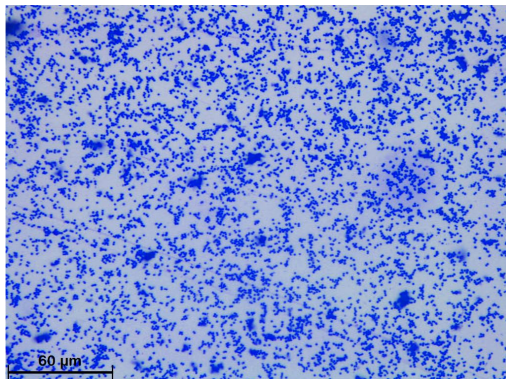


Figure 5.3: Image of MRSA crystal violet staining by stereo microscope

5.4 AGAR WELL DIFFUSION

Susceptibility testing was performed to assess whether the plant extract could inhibit the growth of the MRSA isolate. Although disk diffusion is the gold standard confirming the susceptibility the agar well diffusion was performed because of the plants extract low absorption on the disks and agar plates. The zone of inhibition (ZOI) on the maximum concentration (100mg/ml) was measured at $8\text{mm} \pm 0.0$ in diameter and the ZOI was observed till 6.25 mg/ml concentration around $1\text{m} \pm 0.3$ in diameter. This clearly showed that the plant extract has the ability to inhibit MRSA growth.

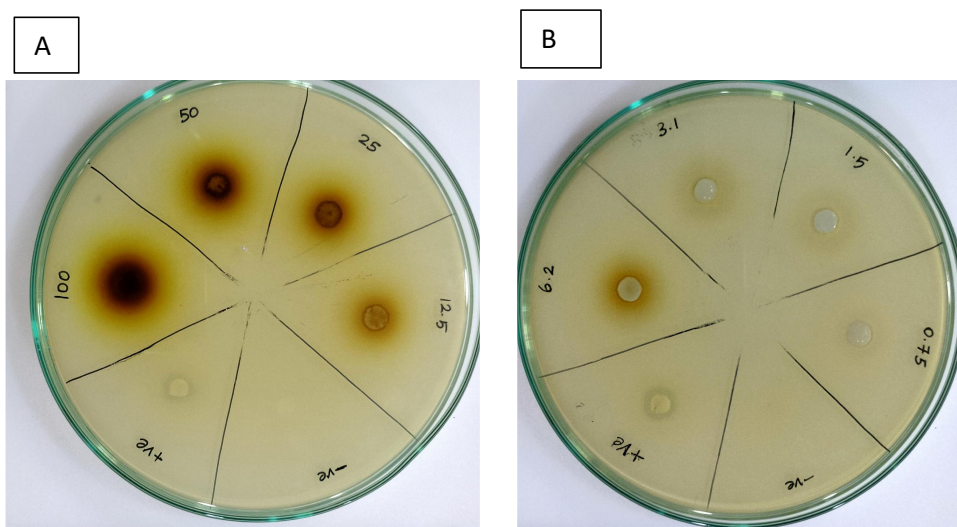


Figure 5.4: Agar well diffusion method with serially diluted plant extract on MRSA

(A) Positive control (Amoxicillin and potassium clavulanate), negative control (solvent-water) (i) 100 mg/ml, (ii) 50 mg/ml, (iii) 25 mg/ml, (iv) 12.5 mg/ml.

(B) Positive control (Amoxicillin and potassium clavulanate), negative control (solvent-water) (i) 6.25 mg/ml, (ii) 3.1 mg/ml, (iii) 1.5 mg/ml, (iv) 0.5 mg/ml.

5.5 BROTH MICRODILUTION

The potential inhibitory activity of the extract was detected against the MRSA. The MIC test result showed that the extract has inhibitory effects on the MRSA ranging from 105 µg/ml to 1000 µg/ml. The MBC test results showed that the extract have bactericidal activity on the MRSA ranging from 500 µg/ml to >2 mg/ml. Also, the MBC result inveterate their bactericidal activity, which is generally two to four times greater than their corresponding MIC values.

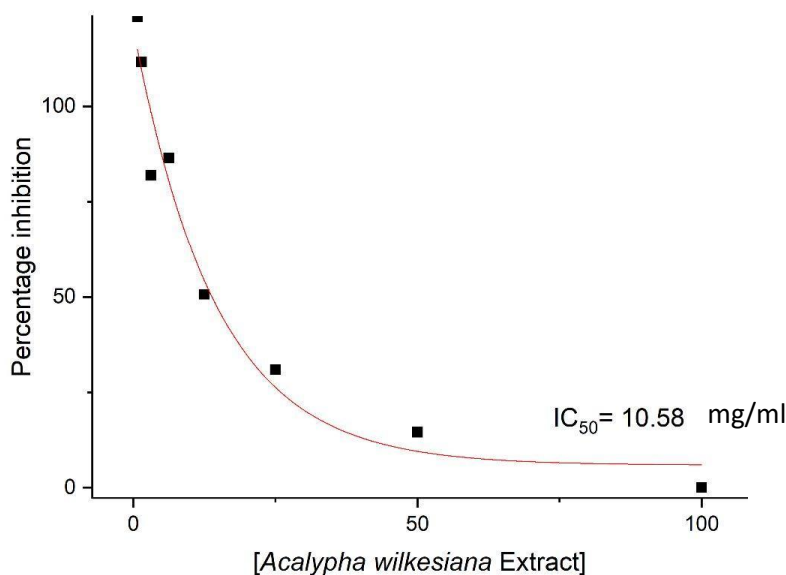


Figure 5.5: The minimum inhibitory concentration (MIC) graph

5.6 TIME KILL ASSAY

The time kill kinetics profile of extract *Acalypha wilkesiana* against MRSA showed reduction in number of viable cells 4,6 and 8 hours respectively, followed by a gradual rise up to the 24th hour when compared to the control.

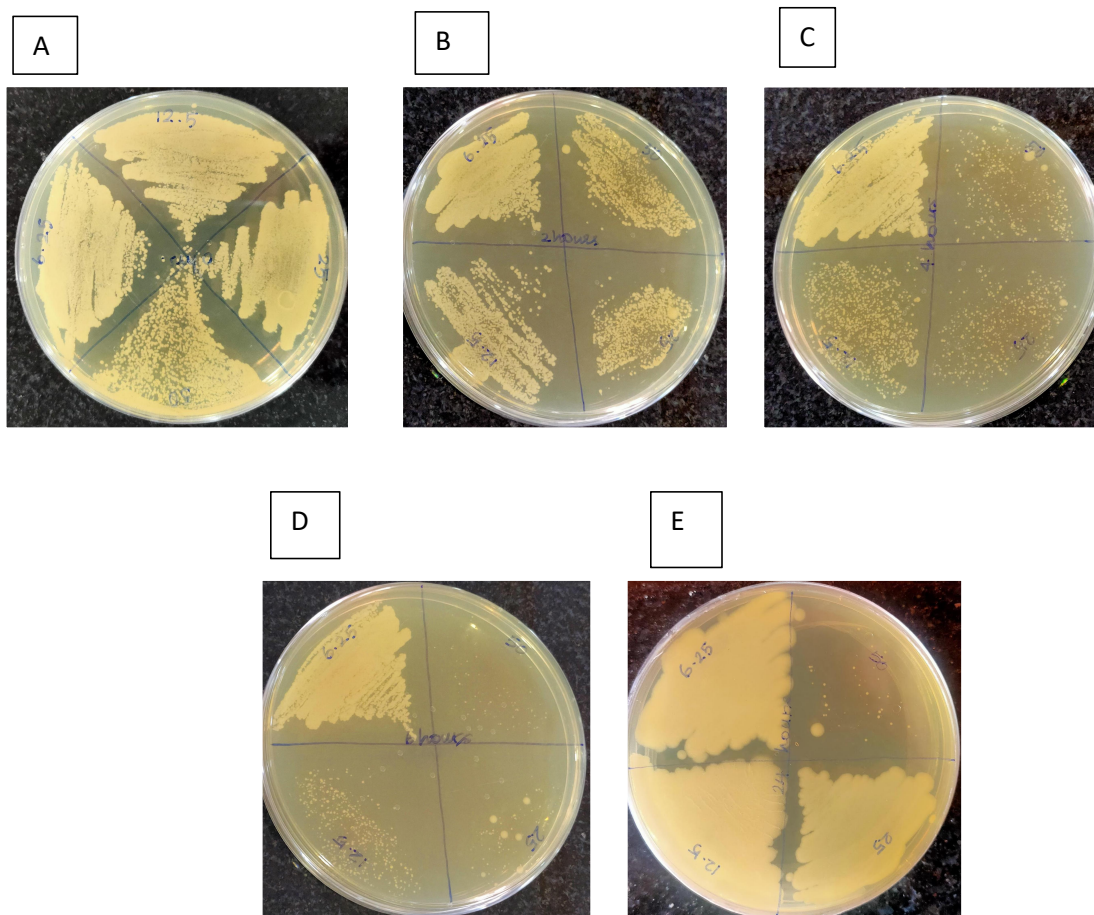


Figure 5.6: Time-kill of assay of *A. Wilkesiana* extract on different time intervals

(A) – 0th hour, (B) – 2nd hour, (C) – 4th hour, (D) – 6th hour and (E) – 24th hour and concentrations (i) 6.25 mg/ml, (ii) 12.5 mg/ml (iii) 25 mg/ml and (iv) 50 mg/ml

Table 5.1: Manual counting of colony forming units on different concentration of extract in different time intervals

Time/no of colonies	6.25 mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml
0 th	TMTC	TMTC	TMTC	950
2 nd	TMTC	TMTC	760	720
4 th	TMTC	732	482	452
6 th	TMTC	420	202	180
24 th	TMTC	TMTC	TMTC	TFTC



Figure 5.7: Graph on the time kill kinetics of the extract in different time intervals

Table 5.2: ImageJ quantification of area on different concentration of extract in different time intervals.

Time (hours)	6.25 mg/ml per mm2	12.5 mg/ml per mm2	25 mg/ml per mm2	50 mg/ml per mm2
0 th	503614	359161	233189	231621
2 nd	464381	336927	219186	210505
4 th	432408	181772	59811	56398
6 th	397009	14830	9115	2066
24 th	493668	455391	392379	6098

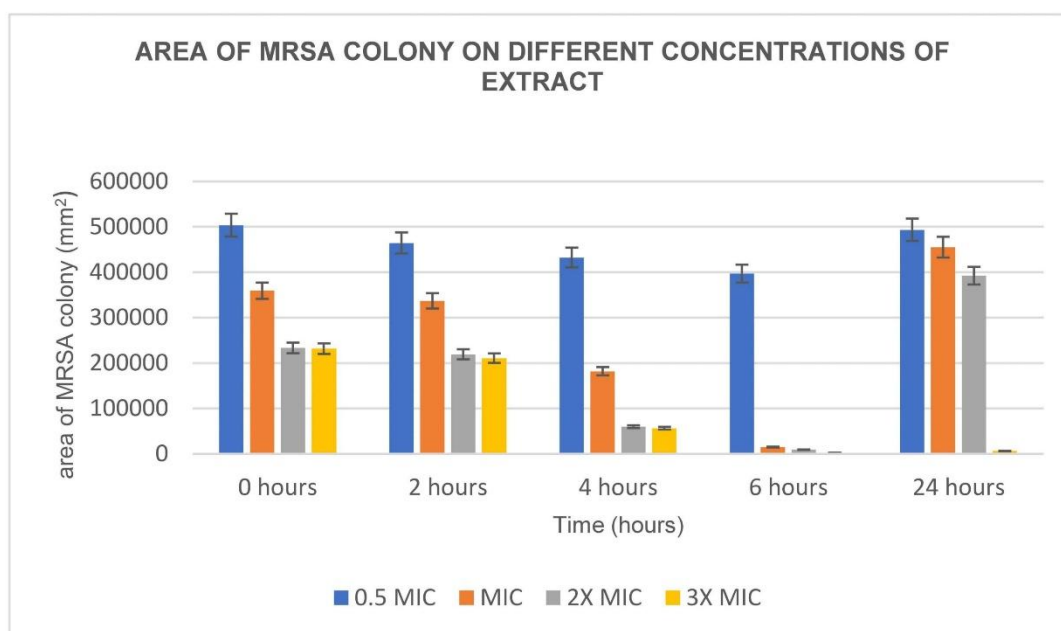


Figure 5.8: Area of MRSA colony on different concentrations of extract on different time intervals

5.7 BIOFILM FORMATION

The period for the formation of biofilm was assessed through a light microscope at different time intervals (24 and 48 h). Different stages in the formation of biofilm are attachment, aggregation, and mat formation. In this study, at 24h of incubation, the planktonic cells get attached to the abiotic surface. At 36 h the cells start to aggregate and at 48 h of incubation, the complete biofilm was formed. The observed results revealed that the ability of the bacteria to form biofilm was influenced by the increased time duration of the incubation period.

Anti-biofilm formation

The anti-biofilm effect of the extract was evaluated both qualitatively and quantitatively. The crystal violet staining method is at three different concentrations such as 12.5 mg/ml, 25 mg/ml, 50 mg/ml. Light microscopy was used as a qualitative method. In the control, most of the areas were covered with a biofilm comprising of clusters of bacteria. Non-aggregation of the cells was 12.5 mg/ml and 25 mg/ml (**figure 5.7 B & C**). In the 50 mg/ml concentration (**figure 5.8**) all the cells were washed off, only stain was left. The quantitative method was using the ImageJ software analysis, done by measuring the area biomass of the biofilm.

Table 5.3: *ImageJ quantification on area of biofilm*

Concentration (mg/ml)	Area/mm2
Control	691666
12.5	395902
25	365995
50	0

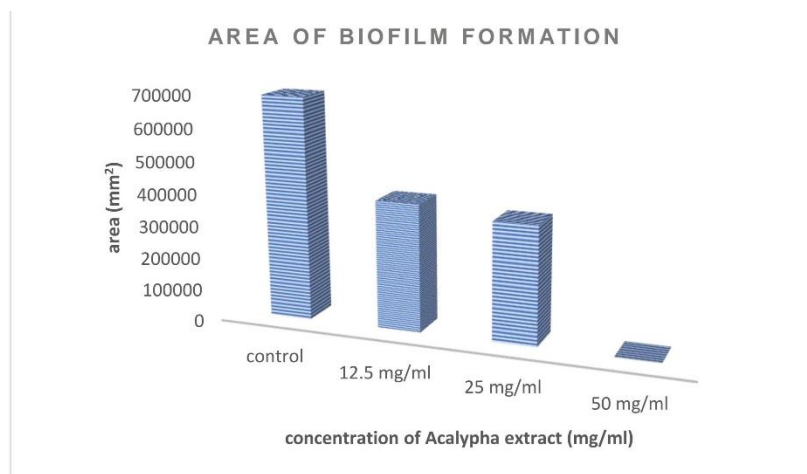


Figure 5.9: 3D Bar graph showing areas of MRSA Biofilm formation

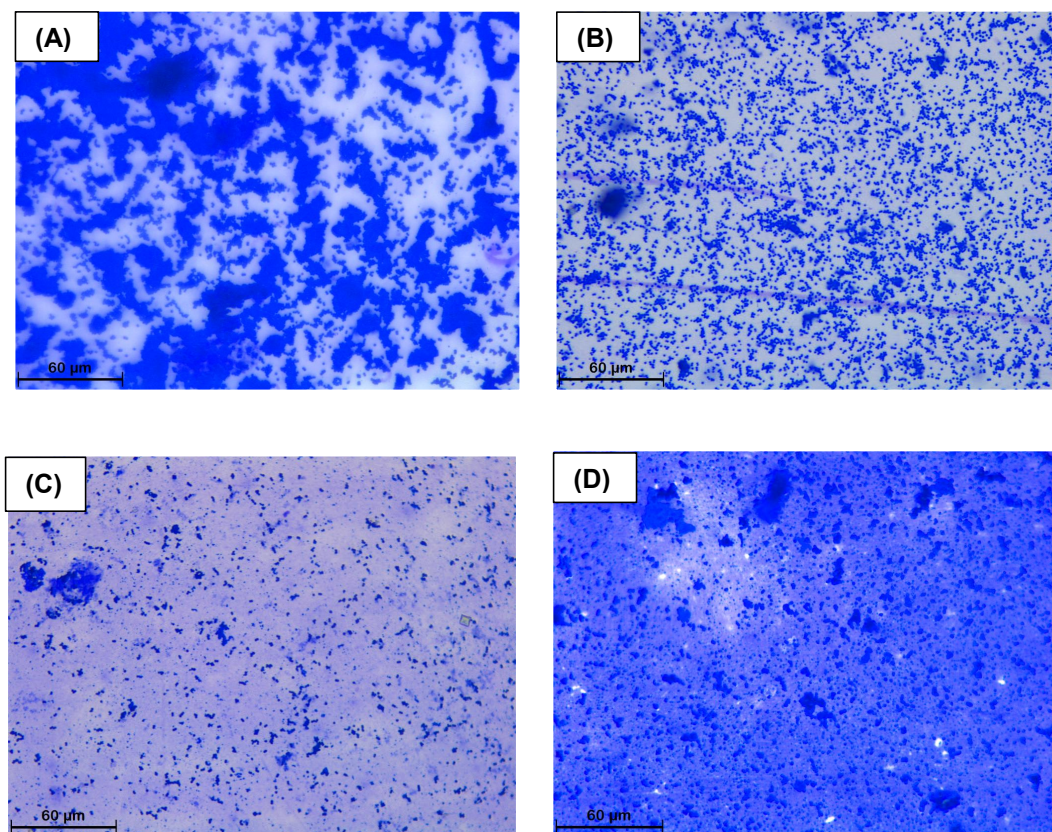


Figure 5.10: Anti-biofilm forming capacity of the *Acalypha wilkesiana*

(A)- control, (B)- 12.5mg/ml, (C)- 25mg/ml, (D)- 50mg/ml

5.8 TOXICITY ASSAY ON ZEBRAFISH EMBRYO AND LARVAE

The Zebrafish acute toxicity test is applied as it establishes the dose that is lethal to 50% of the zebrafish embryos (LC 50) as a sign of acute fish toxicity after a brief exposure to a drug i.e., 96 hours. Usually, an embryo coagulation, lack of somite development, non-detachment of the tail, and/or the absence of a heartbeat are the indications of an embryo's death. In this study observed that approximately 2% of embryos in the highest concentration (100 µg/ml) (**figure 5.8 A**) lacked the somite development which could be determined to be the No observed adverse effect level (NOAEL). In the following concentration (50 µg/ml) (**figure 5.9 B**) there seemed to be a behavioural change with a mild pericardial edema determined as Lowest Observed Effect Level (LOEL). From the next serially diluted concentrations (25 µg/ml to 0.75 µg/ml) (**figure 5.10**) the larvae were observed to be healthy so noted as the no observed effect concentrations (NOEC). Thus, with the observation on the toxicity it would be assumed that the plant extract has a very low toxicity range and so it could be studied on the human cell lines for an accurate toxicity range of the extract of *Acalypha wilkesiana*.

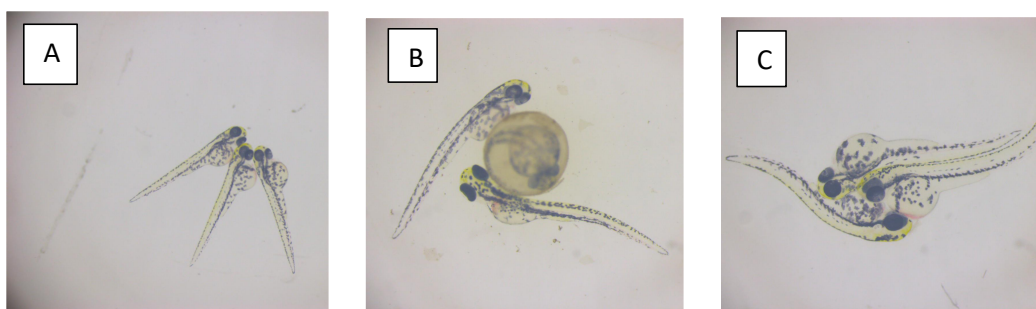


Figure 5.11: Development of zebrafish embryo to larvae, treated with highest concentrations of *A.W* extract

(A) Control- contains only E3 media

(B) 100µg/ml of *A. Wilkesiana* extract in E3 media

(C) 50 µg/ml of *A. Wilkesiana* extract in E3 media

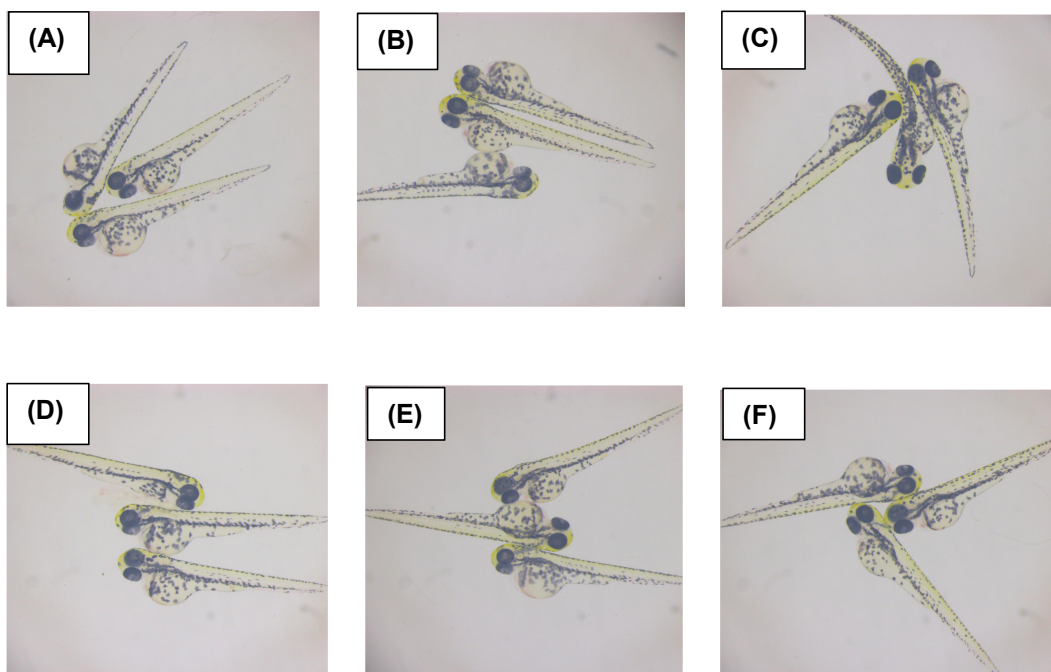


Figure 5.12: No observed effect concentrations (NOEC) in zebrafish larvae treated with serially diluted *A.W* extract

(A) 25 $\mu\text{g/ml}$ (B) 12.5 $\mu\text{g/ml}$ (C) 6.25 $\mu\text{g/ml}$ (D) 3.15 $\mu\text{g/ml}$ (E) 1.5 $\mu\text{g/ml}$ (F) 0.75 $\mu\text{g/ml}$ (Concentration of *A.wilkesiana* extract in E3 media)

CHAPTER 6

SUMMARY AND CONCLUSION

The infections by antimicrobial-resistant pathogens are resulting in worsening and deterioration of health conditions leading to death due to the limited and lack of treatment options. The threat of MRSA infections is escalating, not only by the occurrence of multi-drug resistance but also by the emergence of bacteria that forms a strong biofilm. Since it is one of the main causes of drug resistance, the formation of biofilms by pathogenic microbes has emerged as a serious threat to humanity. There are several reported surveys on antimicrobial activity, but there isn't any reliable research on how it affects the development of bacterial biofilms. The primary emphasis of this work is on the antibacterial and anti-biofilm capacity of the plant *Acalypha wilkesiana* against the human selective pathogenic bacterium, MRSA. The growth of biofilm formation of the MRSA strain was optimized at various time intervals; a full biofilm formation was seen after 48 hours. After that, the inhibitory study was carried out by combining the extract with the culture. This showed better antimicrobial and anti-biofilm activity. The ZFET revealed that the extract is non-toxic and has minimal perniciousness on its maximum concentration. However, various elements of the crude extract provide antibacterial activity. It is additionally important to isolate and purify the specific phytochemical compounds that contribute to the antibacterial and anti-biofilm activities. So, the above observation could promote an alternative promising treatment for the human selective pathogenic organism MRSA.

CHAPTER 7

REFERENCES

1. 2000. Antimicrobial Constituents of the Leaves of *Acalypha wilkesiana* and
2. *Acalypha hispida*. *Pythotherapy Research* Vol. 14, 371-374.
3. Adesina, S.K., Idowu, O., Ogundaini, A.O., Oladimeji, H. and Olugbade, T.A.,
4. Akinyemi, Kabir O., Olukayode Oladapo, Chidi E. Okwara, Christopher C. Ibe, and Kehinde A. Fasure. "Screening of crude extracts of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity." *BMC complementary and alternative medicine* 5 (2005): 1-7.
5. Algammal, Abdelazeem M., Helal F. Hetta, Amr Elkelish, Dalal Hussien H. Alkhalifah, Wael N. Hozzein, Gaber El-Saber Batiha, Nihal El Nahhas, and Mahmoud A. Mabrok. "Methicillin-Resistant *Staphylococcus aureus* (MRSA): one health perspective approach to the bacterium epidemiology, virulence factors, antibiotic-resistance, and zoonotic impact." *Infection and Drug Resistance* (2020): 3255-3265.
6. Asian countries: an ANSORP study. *Journal of Antimicrobial Chemotherapy*.
7. Atoum, Manar F., Hazem Akel, and Mohamed N. Battikhi. "Comparison of PCR and disc diffusion methods in detecting methicillin resistance among *Staphylococcus* species from nosocomial infections." *Saudi medical journal* 24, no. 12 (2003): 1410-1412.
8. Badami, S. And Channabasavaraj, K.P., 2007. In Vitro. Antioxidant Activity of Thirteen Medicinal Plants of India's Western Ghats. *Pharmaceutical biology* 45, 392-396.
9. Basri, Dayang Fredalina, and Radhiah Khairon. "Pharmacodynamic interaction of *Quercus infectoria* galls extract in combination with vancomycin against MRSA

using microdilution checkerboard and time-kill assay.” Evidence-Based Complementary and Alternative Medicine 2012 (2012).

10. Basri, Dayang Fredalina, Nur Azmina Yahya, and Nur Amira Mohd Shamsuddin. “Time-kill assay and post-antibiotic effect of acetone extract from the stem bark of *Canarium odontophyllum* against Methicillin-resistant *Staphylococcus aureus* Mu50 strain.” *Journal of Applied Biology and Biotechnology* 7, no. 3 (2019): 12-16.
11. Canales, M., Hernández, T., Rodriguez-Monroy, M., Flores, C., JiménezEstrada, M., Hernández, L., Durán, A., Hernández-Moreno, M., Trejo, N. And
12. Coates, A.R.M., Halls, G. And Hu, Y., 2011. Novel classes of antibiotics or
13. Davies, J. And Davies, D., 2010. Origins and evolution of antibiotic resistance.
14. Discovery. *Current Opinion in Chemical Biology* 12, 306-317
15. Gajdács, Márió. “The continuing threat of methicillin-resistant *Staphylococcus aureus*.” *Antibiotics* 8, no. 2 (2019): 52.
16. Ganesan, A., 2008. The impact of natural products upon modern drug
17. Haruna, Muyideen T., Chinedu P. Anokwuru, Abosede A. Akeredolu, Adenike A. Akinsemolu, and Okunola A. Alabi. “Antibacterial and antifungal activity of *Acalypha wilkesiana*.” *European Journal of medicinal plants* 3, no. 1 (2013): 52-64.
18. Hernández, A., 2011. Evaluation of the antimicrobial activity of *Acalypha Monostachya* Cav. (Euphorbiales: Euphorbiaceae). *African Journal of Pharmacy and Pharmacology* 5, 640-647.
19. Iyekowa, O., A. P. Oviawe, and J. O. Ndiribe. “Antimicrobial Activities of *Acalypha Wilkesiana* (Red *Acalypha*) Extracts in Some Selected Skin Pathogens.” *Zimbabwe Journal of Science and Technology* 11, no. 1 (2016): 48-57.
20. J.S., Kim, S.W., Chang, H.H. and Kim, Y.S., 2011. Spread of methicillinresistant *Staphylococcus aureus* between the community and the hospitals in
21. Kranjec, Christian, Kirill V. Ovchinnikov, Torstein Grønseth, Kumar Ebineshan, Aparna Srikantam, and Dzung B. Diep. “A bacteriocin-based antimicrobial

- formulation to effectively disrupt the cell viability of methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms.” *Npj Biofilms and Microbiomes* 6, no. 1 (2020): 58.
22. Lade, Harshad, and Jae-Seok Kim. “Bacterial targets of antibiotics in methicillin-resistant *Staphylococcus aureus*.” *Antibiotics* 10, no. 4 (2021): 398.
 23. Lakhundi, Sahreena, and Kunyan Zhang. “Methicillin-resistant *Staphylococcus aureus*: molecular characterization, evolution, and epidemiology.” *Clinical microbiology reviews* 31, no. 4 (2018): e00020-18.
 24. Lee, Andie S., Hermínia De Lencastre, Javier Garau, Jan Kluytmans, Surbhi Malhotra-Kumar, Andreas Peschel, and Stephan Harbarth. “Methicillin-resistant *Staphylococcus aureus*.” *Nature reviews Disease primers* 4, no. 1 (2018): 1-23.
 25. Mabrouk, Mostafa, Rasha M. Abd El-Wahab, Hanan H. Beherei, Mohamed M. Selim, and Diganta B. Das. “Multifunctional magnetite nanoparticles for drug delivery: Preparation, characterisation, antibacterial properties and drug release kinetics.” *International Journal of Pharmaceutics* 587 (2020): 119658.
 26. Mastoor, Sobia, Fizza Nazim, Syed Rizwan-ul-Hasan, Khalid Ahmed, Shabnam Khan, Syed Nawazish Ali, and Syed Hani Abidi. “Analysis of the Antimicrobial and Anti-Biofilm Activity of Natural Compounds and Their Analogues against *Staphylococcus aureus* Isolates.” *Molecules* 27, no. 20 (2022): 6874.
 27. *Microbiology and Molecular Biology Reviews* 74, 417-433.
 28. More of the same? *British Journal of Pharmacology* 163, 184-194.
 29. Nwabuife, Joshua C., Calvin A. Omolo, and Thirumala Govender. “Nano delivery systems to the rescue of ciprofloxacin against resistant bacteria “*E. Coli*; *P. Aeruginosa*; *Saureus*; and MRSA” and their infections.” *Journal of Controlled Release* 349 (2022): 338-353.
 30. Pantosti, A., and M. Venditti. “What is MRSA?.” *European Respiratory Journal* 34, no. 5 (2009): 1190-1196.

31. Peacock, Sharon J., and Gavin K. Paterson. "Mechanisms of methicillin resistance in *Staphylococcus aureus*." *Annual review of biochemistry* 84 (2015): 577-601.
32. Prenafeta, Antoni, M. Sitjà, Mark A. Holmes, and Gavin K. Paterson. "Biofilm production characterization of *mecA* and *mecC* methicillin-resistant *Staphylococcus aureus* isolated from bovine milk in Great Britain." *Journal of Dairy Science* 97, no. 8 (2014)
33. Shriram, Varsha, Tushar Khare, Rohit Bhagwat, Ravi Shukla, and Vinay Kumar. "Inhibiting bacterial drug efflux pumps via phyto-therapeutics to combat threatening antimicrobial resistance." *Frontiers in microbiology* 9 (2018): 2990.
34. Silva, Danielle M., PRISCILLA A. COSTA, Andrea OB Ribon, Gislaine A. Purgato, Diaz-Muñoz Gaspar, and Marisa AN Diaz. "Plant extracts display synergism with different classes of antibiotics." *Anais da Academia Brasileira de Ciências* 91 (2019).
35. Song, J.H., Hsueh, P.R., Chung, D.R., Ko, K.S., Kang, C.I., Peck, K.R., Yeom,
36. Umamaheswari, A., and Grace Felciya SJ. "Investigation on the biofilm eradication potential of selected medicinal plants against methicillin-resistant *Staphylococcus aureus*." *Biotechnology Reports* 28 (2020): e00523.